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13. ABSTRACT (Maximum 200 Words)

NY-ESO-1 is expressed in 40% of malignant breast tumors and is a promising antigen for breast cancer immunotherapy. Recombinant *Listeria monocytogenes* expressing specific target antigens is a highly effective vaccine vector, inducing cell-mediated immunity capable of regressing established tumors. The full-length and partial sequences (residues 1-108, 101-156 and 148-180) of NY-ESO-1 were cloned as fusion genes with listeriolysin-O (LLO) and expressed in Listeria. Secretion of the fusion proteins from constructs containing the hydrophobic C-term region of NY-ESO-1 was poor or absent. Vaccination of mice with *Listeria* expressing either the full-length NY-ESO-1 or its fragments did not significantly impact tumor growth of the mammary carcinoma 4T1 cell line, which was engineered to express NY-ESO-1. We observed that these *Listeria* constructs were deficient in intracellular proliferation in murine J774 cells, and are highly attenuated in mice. New constructs were made, fusing NY-ESO-1 to the LLO PEST sequence only, which is an immunologically important domain in LLO. The PEST-NY-ESO-1 constructs showed a better proliferation in J774 cells between 8 and 24 hours. We are also trying to express NY-ESO-1 under the control of the listerial actA promoter, which is activated in the cytosol of the infected cell. These new constructs are currently being tested in animal studies.

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I. Introduction

Breast cancer is the most common malignancy in women. In U.S., 180,000 new cases are diagnosed each year, in addition to 45,000 deaths caused by breast cancer. Current therapy for this disease (such as surgery, radiation and chemotherapy) is aggressive and in some cases mutilating. More recently, new developments in tumor antigen identification and immune activation raised interest in cancer immunotherapy, which is specific against the cancer cells and with fewer side effects.

The initial goal of this project was to develop a *Listeria monocytogenes*-based vaccine against the Her2/neu antigen, which is overexpressed in a high percentage of breast cancers. As previously reported, several Listeriolysin-O (LLO)-Her2/neu fusion proteins were constructed and evaluated in animal models in our laboratory. *L. monocytogenes* has been successfully used as a vaccine vector and tested in several disease models. This gram-positive facultative intracellular bacterium preferentially infects antigen-presenting cells (APC), such as macrophages and dendritic cells (DC), triggering a strong cell-mediated immune response, stimulating both CD8 and CD4 T cells. Most importantly, these responses are extended to the passenger antigen expressed by a recombinant *Listeria* vector (Weiskirch and Paterson, 1999). In fact, vaccination with recombinant *Listeria* expressing target antigens is able to cure mice affected with established tumors, through activation of strong cell mediated immunity (Pan *et al.*, 1995 and 1999, Gunn *et al.*, 2001). A unique characteristic of *L. monocytogenes* is its ability to escape to the cytosol from the vacuole upon secretion of the LLO virulence factor, allowing *L. monocytogenes*-encoded antigens to reach both the MHC class I and class II antigen-presentation pathways.

To improve our immunotherapeutic approach to breast cancer, it is important we increase the scope of breast tumor antigens represented in our *Listeria* vaccines. Although Her2/neu is a potential candidate for breast cancer vaccines, it is a self protein and vaccination regimens have shown only moderate success. In addition, it is now widely accepted that tumors can escape immunotherapeutic strategies that target a single antigen by losing expression of that antigen. A promising group of tumor antigens is the so-called cancer/testis (CT) antigens, which are universally expressed in testis and also in a wide range of different tumors. NY-ESO-1 is a member of the CT antigen family, whose gene encodes for a protein with 180 residues (Chen *et al.*, 1997). Immunohistochemical analysis and mRNA detection of NY-ESO-1 revealed that this antigen is expressed in 20 to 30% of lung, bladder and ovarian cancers and melanoma (Jungbluth *et al.*, 2001). In breast tumors, Sugita *et al.* (2004) detected the NY-ESO-1 mRNA in 42% and 68% of the specimens from malignant and benign tumors, respectively. NY-ESO-1 is the most immunogenic CT antigen described so far and antibodies against it are found in 40 to 50% of the patients with NY-ESO-1-expressing tumors (Stockert *et al.*, 1998). Interestingly, more than 90% of the patients with antibodies against NY-ESO-1 also develop a specific $CD8^+$ T-cell response for this antigen (Jäger *et al.*, 2000). In breast cancers, a higher rate of NY-ESO-1 expression was observed among tumors with high histological grade and negative hormone receptor status, suggesting that NY-ESO-1 could be a potential tumor antigen for immunotherapy in those cases with a poor prognosis (Sugita *et al.*, 2004).

II. Body

Her2/neu and NY-ESO-1 are two potential candidates for immunotherapy of breast cancer, especially in patients with a poorer prognosis. Additionally, it is likely that a combination of these two antigens provides a more efficient therapeutic approach. Furthermore, NY-ESO-1 has some advantages, as a high immunogenicity and expression in normal tissues restricted to testis, although sometimes a low expression can also be detected in ovary and placenta.

As described in the first report, we generated recombinant *L. monocytogenes* expressing the NY-ESO-1 gene and also fragments of it fused to the listerial protein LLO (Figure 1). Analysis of secretion of the recombinant proteins by Western-blot revealed that only the constructs LLO-NY-ESO-1/1-108 and 101-156 were strongly secreted, whereas the construct LLO-NY-ESO-1/1-180 had a weak secretion. On the other hand, the fusion proteins LLO-NY-ESO-1/101-180 and 148-180, which contain the C-term region of NY-ESO-1, are poorly secreted (Figure 1). One possible cause is the high hydrophobicity of this region.

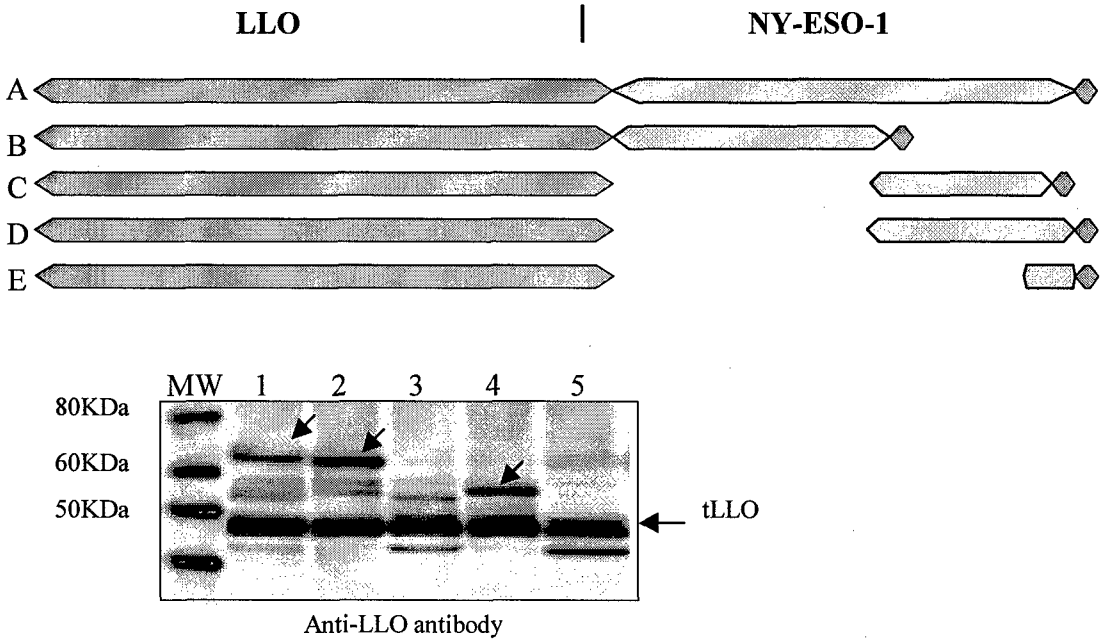

Figure 1. Upper: LLO-NY-ESO-1 fusion proteins constructed and tested for expression and secretion in *Listeria monocytogenes*. A) LLO-NY-ESO-1/1-180; B) LLO-NY-ESO-1/1-108; C) LLO-NY-ESO-1/101-156; D) LLO-NY-ESO-1/101-180; E) LLO-NY-ESO-1/148-180. Bottom: Western-blot of LLO-NY-ESO-1 constructs. MW: molecular weight marker; lane 1: LLO-NY-ESO-1/1-180 (~70KDa); lane 2: LLO-NY-ESO-1/1-108 (~62KDa); lane 3: LLO-NY-ESO-1/101-180 (~58KDa); lane 4: LLO-NY-ESO-1/101-156 (~56KDa); lane 5: LLO-NY-ESO-1/148-180 (~54KDa). A band of 48KDa, corresponding to the truncated LLO (tLLO), is observed.

We generated mouse tumor cell lines that constitutively express the human NY-ESO-1 gene. As a breast tumor model, we chose the 4T1 cell line, which is a cell line derived from a BALB/c mammary carcinoma. The 4T1 mammary carcinoma model is also very useful to evaluate the effect of the vaccine in a metastatic breast disease, since these cells spontaneously metastasize (Pulaski and Ostrand-Rosenberg, 1998). We used a retroviral-based system to

transduce this cell line with the human NY-ESO-1 gene, whose expression in this model is under the control of the human Ubiquitin C promoter. Expression of NY-ESO-1 was verified by Western-blot in several clones (Figure 2). In general, the expression of NY-ESO-1 by the different clones was weak in this cell line, although all the clones tested expressed the antigen.

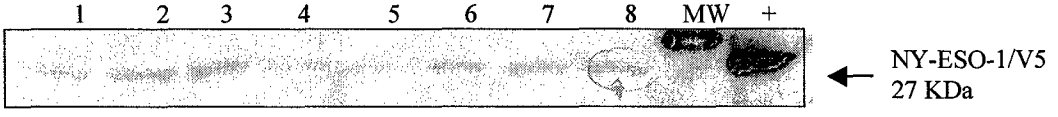

Figure 2. Western-blot of 4T1 cell line clones transduced with NY-ESO-1, using a rabbit polyclonal antibody specific against the NY-ESO-1 protein. Lanes 1 to 8 represent different clones selected after transduction; MW, molecular weight marker and (+) is a positive control for NY-ESO-1/V5 protein.

One of the clones was selected, based on morphology, antigen expression and tumorigenicity in vivo, for further studies. To evaluate if our vaccine could impact the tumor growth of the NY-ESO-1-expressing 4T1 cell line, we inject $1x10^5$ cells per mouse on day 0, and vaccinated the animals on days 5, 12 and 19 with $2.5x10^8$ CFU of our *Listeria* vaccine expressing the full-length NY-ESO-1 fused to LLO. This initial study showed no effect of our vaccine on tumor growth (Figure 3). In the NY-ESO-1 group, two mice had smaller tumors at the primary site, however they had ascites and died earlier due to metastasis. The negative control was an irrelevant antigen, cloned into *Listeria* in the same way as NY-ESO-1. We also tested the LLO-NY-ESO-1/1-108 and the LLO-NY-ESO-1/101-156 constructs and observed the same result (data not shown).

The *Listeria*-based vaccines against NY-ESO-1 we tested were highly attenuated. The LD_{50} determination for all constructs was above $2.5x10^9$ CFU per mouse. However, at this dose the mice died within 48 hours of toxic shock, instead of listeriosis. In this case, we do not know if our constructs are still able to cause murine listeriosis and maintain some required virulence factors important for immune activation by *Listeria*. We initially evaluated our constructs in cell infection assays, where we infect the murine macrophage-like cell line J774 with *Listeria* and count the number of bacteria in these cells overtime. The extracellular *Listeria* are killed by using medium with gentamicin, and the number of bacteria recovered after lysis of the infected cells is an estimation of the intracellular *Listeria*. This assay showed that our constructs infected J774 cells but were unable to replicate inside the cells, as compared to the wild-type 10403S *Listeria* (Figure 4a). One possible explanation is that our *Listeria* constructs are not escaping the phagolysosome. It has been shown that *Listeria* has to invade the cytosol to generate a $CD8^+$ T cell immune response and these findings can explain the high LD_{50} and why our vaccine initially failed in animal studies. To verify if this was related to the *Listeria* XFL-7 strain we used, we transformed another prfA deficient *Listeria* (DPL1028) with the same NY-ESO-1 constructs. The DPL1028 NY-ESO-1 constructs had the same behavior in J774 cells compared to the XFL-7 (Figure 4b). In this experiment, besides the 10403S, we also include the Lm-LLO-NP construct, which carries the influenza nucleoprotein fused to LLO and it is highly immunogenic *in vivo*.

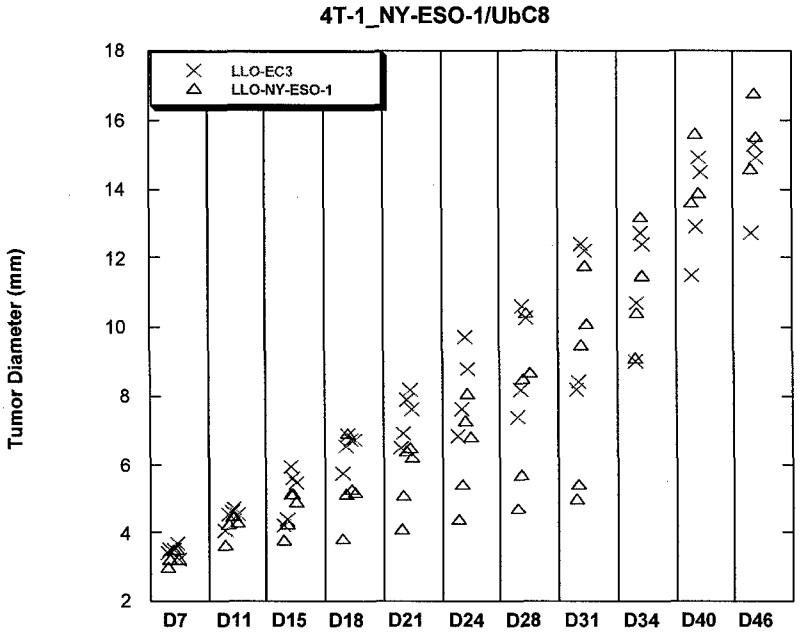

Figure 3.Tumor regression study with the NY-ESO-1-expressing 4T1 cell line.

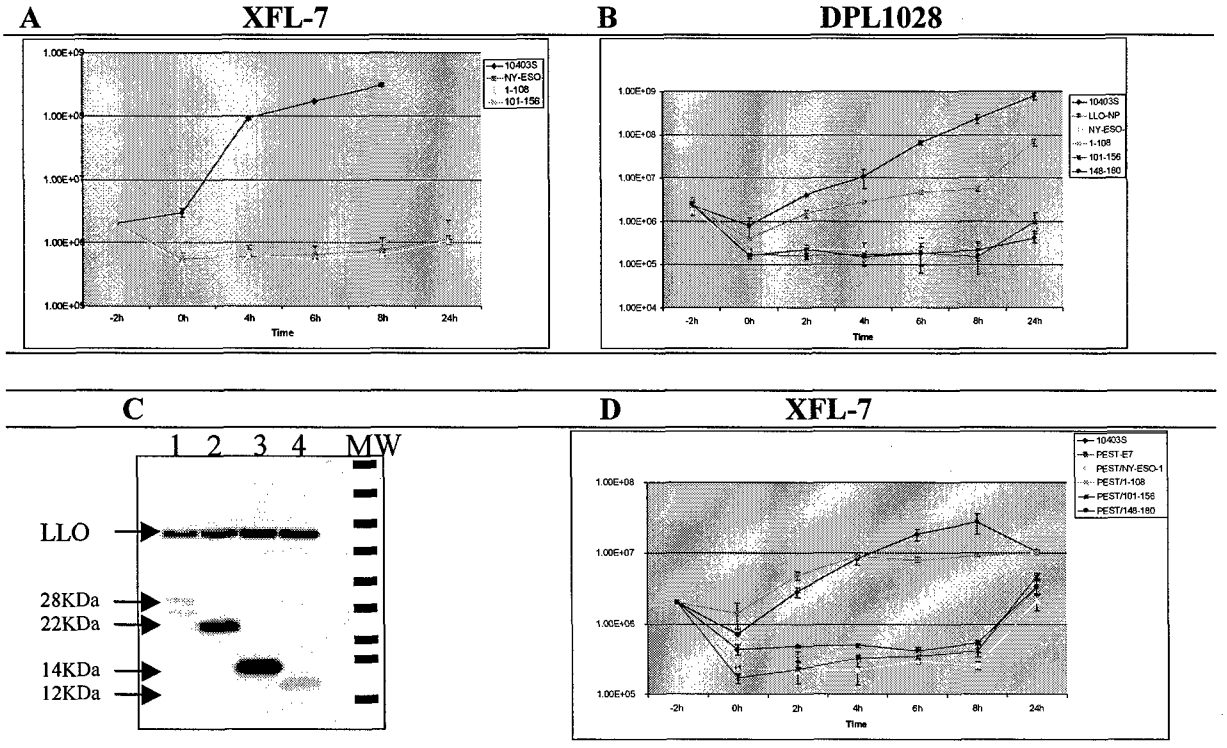

Figure 4. Cell infection assay in J774 cells using the prfA⁻ Listeria strains XL-7 (A and D) and DPL1028 (B) expressing NY-ESO-1 fused to either LLO (A and B) or PEST (D). C) Western-blot to demonstrate secretion of the PEST-NY-ESO-1 fusion proteins: 1) PEST-NY-ESO-1/1-180 (~28KDa); 2) PEST-NY-ESO-1/1-108 (~22KDa); 3) PEST-NY-ESO-1/101-156 (~14KDa); 5) PEST-NY-ESO-1/148-180 (~12KDa). A band of 58KDa, corresponding to the endogenous LLO is observed.

Based on this information, new constructs were proposed to make them less attenuated. The first attempt was to clone the NY-ESO-1 gene and its fragments in fusion with the first 50 residues from the LLO sequence, which contain the important PEST domain of this protein (Decatur and Portnoy, 2000). The idea was to relieve the bacteria from producing the truncated LLO present in the plasmid, enhancing the production of the endogeneous full-length LLO protein. Expression and secretion of the PEST-NY-ESO-1 constructs were demonstrated in *Listeria* (Figure 4c). As expected, we also observed a strong 58 KDa band corresponding to the endogeneous LLO protein. However, in J774 cell infection assay, these PEST constructs did not perform as well as expected, showing a behavior similar to the previous constructs, except that a better recovery of colonies was observed at 24 hours (Figure 4d).

We also considered expressing NY-ESO-1 under the control of the listerial actA promoter (PactA), which is activated in the cytosol of the infected cell. In this case, the expression of our NY-ESO-1 construct would not take place in the phagolysosome, avoiding any deleterious effect of the fusion protein in *Listeria* escape to the cytosol. Additionally, this system is being made in a new listeria strain (Dal$^-$/Dat$^-$), which is deficient in the alanine racemase gene. In this system, our fusion gene is cloned in frame to LLO into a plasmid (pTV3) carrying the Dal gene to complement the deficient *Listeria* strain (Verch *et al.*, 2004). This has been successfully tested in our lab with the HPV16 E7 antigen. After replacing the hly promoter by the PactA in pTV3, we analyzed the expression in vitro of the LLO-E7 fusion protein. In this experiment, we grew the cells in BHI medium with or without 5% activated charcoal. The PactA is inactive in BHI medium, unless activated charcoal is added. However, the Western-blot showed that the PactA was constitutively activated, even in BHI without charcoal, although an increase in activity was clearly detected in the presence of charcoal (Figure 5a). The PactA activation is strictly dependent on the presence of the prfA transcription activator, whose gene is also cloned in the pTV3 plasmid. In this case, the overexpression of prfA from the plasmid could explain the loss of regulation of the PactA in this *Listeria*. To solve this, we removed a 1.6 Kb fragment containing the prfA gene from pTV3(PactA) plasmid (Figure 5b). Currently we are testing if we can reestablish the PactA regulation in this plasmid.

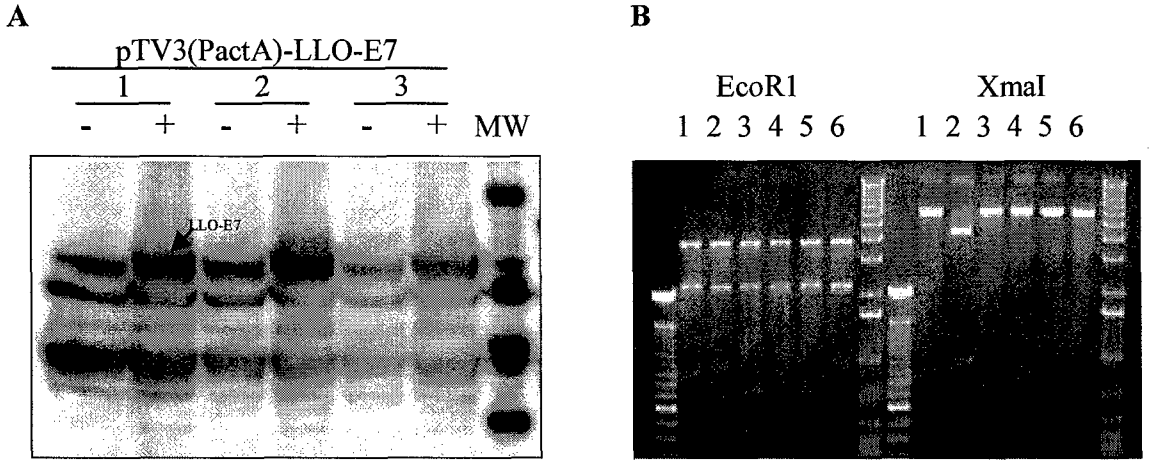

Figure 5. A. Western-blot of 3 different clones of *L. monocytogenes* (Dal-/Dat-) expressing LLO-E7 under the control of the actA promoter in the presence (+) or absence (-) of 5% activated charcoal; B. pTV3 plasmid after excision of the prfA gene and digested with either EcoRI (3.8Kb and 2.2Kb bands expected) or XmaI (6Kb band expected) enzymes. Clone 2 lost the XmaI site used in the digestion/ligation during the cloning procedure.

Once we have characterized this construct with the PactA promoter, we intend to test its efficacy using the well established E7 model in our lab, and subsequently replace the E7 antigen by the NY-ESO-1 gene and its fragments. These new constructs will be evaluated in future studies for their ability to induce an immune response against NY-ESO-1 antigen and treat established tumors expressing this antigen.

III. Key Research Accomplishments

- Generation of 4T1 cells expressing NY-ESO-1 under the control of the human Ubiquitin C promoter;
- Testing the efficacy of the *Listeria*-based vaccines against the NY-ESO-1 antigen, using the 4T1 mammary tumor as a model;
- Cloning of the LLO-NY-ESO-1 constructs in another prfA$^-$ *Listeria* strain, known as DPL1028;
- Generation of new *Listeria* constructs fusing only the PEST domain of LLO to the NY-ESO-1 gene and its fragments;
- Studying the replication of the several *Listeria*-NY-ESO-1 constructs in cell infection assays using J774 cells;
- Replacement of the hly promoter by the actA promoter in the pTV3 plasmid and analysis of the actA promoter activity in this plasmid;
- Removal of the prfA gene from the pTV3 plasmid.

IV. Reportable Outcomes

Part of this work was presented in October 2004 in the *Cancer Vaccines 2004,* sponsored by the Cancer Research Institute and the Ludwig Institute for Cancer Research, held in New York, USA

V. Conclusions

In summary, we constructed several recombinant *Listeria monocytogenes* that express NY-ESO-1. We found that the C-term region of NY-ESO-1, which contains the HLA-A2 157-165 epitope, is poorly secreted by *Listeria*. Additionally, vaccination of mice with *Listeria* expressing either the full-length NY-ESO-1 or its fragments did not significantly impact tumor growth of the mammary carcinoma 4T1 cell line, which was engineered to express NY-ESO-1. Further analysis showed that these *Listeria* constructs are deficient in intracellular proliferation in murine J774 cells, and are highly attenuated (LD50 of 2.5×10^9 CFU) in mice. Cloning the NY-ESO-1 gene and its fragment into the prfA$^-$ *Listeria* strain DPL1028, instead of XFL-7, changed neither the secretion of the LLO-NY-ESO-1 fusion proteins nor their proliferation in J774 cells. New constructs were made, fusing NY-ESO-1 to the LLO PEST sequence only, which is an immunologically important domain in LLO. The PEST-NY-ESO-1 constructs did not showed a consistent proliferation in J774 cells in the first 8 hours, although a better proliferation was observed between 8 and 24 hours. We also considered expressing NY-ESO-1 under the control of the listerial actA promoter, which is activated in the cytosol of the infected cell. In this case, the expression of our NY-ESO-1 construct would not take place in the phagolysosome, avoiding any deleterious effect of the fusion protein in *Listeria* escape to the cytosol. A plasmid called

pTV3 has been modified to test the efficiency of the actA promoter in triggering immune responses against tumor antigens under its control. Current therapy for breast cancer, such as radiation and chemotherapy, is aggressive and destroy normal tissue. In contrast, immunotherapeutic approaches are more specific, ideally targeting only tumor cells, and associated with fewer side effects. Furthermore, immunotherapies may offer hope to cancer patients in cases where conventional therapy has failed.

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